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Akt1 isoform modulates phenotypic conversion of vascular smooth muscle cells

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ABSTRACT

In this study, we investigated the role of Akt1 isoform in phenotypic change of vascular smooth muscle cells (VSMCs) and neointima formation. Laminin-induced conversion of synthetic VSMCs into contractile VSMCs was measured by expression of marker proteins for contractile VSMCs and collagen gel contraction assay. Culture of synthetic VSMCs on laminin-coated plates induced expression of marker proteins for contractile VSMCs and showed contraction in response to angiotensin II (AngII) stimulation. Silencing integrin-linked kinase attenuated activation of Akt and blocked phenotypic conversion of VSMCs resulting in the loss of AngII-dependent contraction. Laminin-induced phenotypic conversion of VSMCs was abrogated by phosphatidylinositol 3-kinase inhibitor or in cells silencing Akt1 but not Akt2. Proliferation of contractile VSMCs on laminin-coated plate was enhanced in cells silencing Akt1 whereas silencing Akt2 did not affect. Promoter activity of myocardin and SM22α was enhanced in contractile phenotype and overexpression of myocardin stimulated promoter activity of SM22α in synthetic phenotype. Promoter activity of myocardin and SM22α was reduced in cells silencing Akt1 and promoter activity of SM22α was restored by overexpression of myocardin in cells silencing Akt1. However, silencing of Akt2 affected neither promoter activity of myocardin nor SM22α. Finally, neointima formation in carotid artery ligation and high fat-diet-induced atherosclerosis was facilitated in mice lacking Akt1. This study demonstrates that Akt1 isoform stimulates laminin-induced phenotypic conversion of synthetic VSMCs by regulating the expression of myocardin. VSMCs become susceptible to shifting from contractile to synthetic phenotype by the loss of Akt1 in pathological conditions.

1. Introduction

Vasoconstriction, vascular tone, blood pressure, and blood flow are controlled by the highly specialized vascular smooth muscle cells (VSMCs). VSMCs halt proliferation and express specialized proteins such as a repertoire of contractile proteins and ion channels [1]. In a mature blood vessel, VSMCs display a contractile phenotype characterized by expression of specific marker proteins such as myosin heavy chain (MHC), myosin light chain kinase (MLCK), smooth muscle actin (SMA), SM22α, and calponin, which is an important protein for contraction [1]. Unlike cardiac or skeletal myocytes, terminally differentiated VSMCs retain phenotypic plasticity and undergo reversible changes in response to environmental stimuli. Such processes include loss of the physiological function of VSMCs and play key roles in the neointima formation during pathogenesis of atherosclerosis and restenosis during angioplasty, stenting, or bypass surgery [2].

During embryonic development, contractile VSMCs originate from embryonic stem cells or pericytes. In addition, the differentiation of VSMCs is well characterized *in vitro* using mouse or human embryonic stem cells (ESCs). For example, ESCs differentiate into either endothelial cells (ECs) or VSMCs under appropriate conditions [3]. It also has been reported that expression of myocardin, a cofactor for serum response factor (SRF), plays key roles in the differentiation of VSMCs [4]. Expression of myocardin itself is regulated by variety of transcription factors including Mef2, Tead, and Fox proteins [5]. Once VSMCs acquire differentiated status, they need certain environmental cues to maintain differentiated status. For instance, it has been reported that laminin and insulin-like growth factor-1 (IGF-1) provide the ability of VSMCs to maintain a contractile phenotype under *in vitro* culture conditions [6]. In the absence of such environmental cues, VSMCs rapidly display phenotypic changes [7]. Phenotypic conversion of VSMCs from contractile

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VSMCs into synthetic VSMCs eventually acquires cell cycle re-entry, migration, and extracellular matrix synthesis and thereby cause intimal thickening. Therefore, maintaining contractile phenotypes of VSMCs seems to be important for healthy blood vessels.

The vessel architecture is mainly composed of ECs, VSMCs, adventitial fibroblasts, and extracellular matrix (ECM) molecules. Each cell rests on basement membrane, which is a specialized structure composed of ECM molecules such as laminins, collagens, elastin, and proteoglycans [8]. Especially, the roles of ECM proteins in VSMC plasticity have been investigated. Stimulation of contractile VSMC with plasma fibronectin strongly promoted a phenotypic change [9]. By contrast, culture of contractile VSMCs on laminin-coated dish maintained the contractile phenotype of VSMCs [10]. Therefore, interaction of VSMCs with ECM proteins seems to be an important determinant in the phenotypic changes of VSMCs. However, the signaling cascades involved in ECM-dependent change of synthetic VSMCs into contractile VSMCs are largely unknown.

ECM proteins bind to integrins, which are a large family of glycosylated, heterodimeric, and transmembrane proteins. Each heterodimer consists of one α and one β subunit that associates non-covalently to produce a functional receptor [11]. Once integrins are activated, their cytoplasmic tails can bind numerous proteins, which in turn transduce signaling cascades. Proteins that are involved in integrin signaling include focal adhesion kinase (FAK), integrin-linked kinase (ILK), paxillin, Src family kinases, and others [12]. ILK is a serine/threonine protein kinase that binds to β subunit of integrins. ILK contains a pleckstrin homology (PH) domain that binds phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which activates ILK activity *via* the phosphatidylinositol 3-kinase (PI3K) pathway. ILK also binds Akt and phosphorylates its Ser473 site [13]. ILK can influence a variety of cellular physiologies such as survival, proliferation, migration, differentiation, and epithelial–mesenchymal transition (EMT) [14]. The significance of the ECM-ILK interaction and its downstream signaling pathways in the phenotypic change of VSMCs has not been fully elucidated yet.

Akt is serine/threonine kinase that regulates a variety of cellular responses. The Akt family of enzyme consists of three isozymes (Akt1, Akt2, and Akt3) encoded by three different genes [15]. Although each isoform shares ~85% homology in amino acid sequence, different functions for each isoform have been implicated in targeted mice models [16–18]. However, the role of Akt isoform in the phenotypic change of VSMCs is still ambiguous. In the present study, we investigated the role of the ECM/ILK/Akt1 axis in the regulation of VSMC phenotypes as well as in neointima formation during the pathogenesis of atherosclerosis.

2. Materials and methods

2.1. Materials

Anti-myosin heavy chain (MHC) antibody was obtained from Proteintech Group Inc. (Chicago, IL, USA). Anti-myosin light chain kinase (MLCK) antibody was purchased from Epitomics, Inc. (Burlingame, CA, USA). Antibodies against smooth muscle actin (SMA), calponin, and SM22α were obtained from Sigma-Aldrich (St Louis, MO, USA). Anti-actin antibody was purchased from MP biomedicals (Aurora, OH, USA). Anti-ERK1/2 and anti-p-Akt (Ser473) antibodies were obtained from Cell Signaling Technology (Boston, MA, USA). Antibodies against ILK, pan-Akt, Akt1, and Akt2 were purchased from Millipore Corporation (Billerica, MA, USA). AngII and laminin isolated from human placenta were obtained from Sigma-Aldrich (St Louis, MO, USA). Type I collagen was purchased from BD Biosciences (San Jose, CA, USA). The promoter region of SM22α subcloned in pGL3 vector was a kind gift from Dr. Gary K. Owens (University of Virginia). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless indicated elsewhere.

2.2. Animals

Four-week-old male Sprague–Dawley rats were purchased from Koatech Inc. (Pyungtak, Korea). Mice lacking Akt1 (*Akt1*$^{-/-}$, C57B6.129P2-*Akt1*^{tm1Mbb}/J) and ApoE (*ApoE*$^{-/-}$, C57B6.129P2-*Apoe*^{tm1Unc}/J) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Akt1$^{-/-}$ mice were crossed with ApoE-deficient mice (*ApoE*$^{-/-}$) to generate heterozygous mice at both loci. *Akt1*$^{+/-}$ *ApoE*$^{-/-}$ mice were intercrossed to produce *Akt1*$^{+/+}$*ApoE*$^{-/-}$ and *Akt1*$^{-/-}$*ApoE*$^{-/-}$ littermates. Mice were housed under specific pathogen free (SPF) conditions. All animal procedures were done in accordance with our institutional guidelines for animal research, and were approved by our Institutional Animal Care and Use Committee (PNU-2009-0008). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th edition, 2011).

2.3. Cell preparation, culture, and induction of phenotypic conversion

Vascular smooth muscle cells (VSMCs) were isolated from 4-week-old Sprague–Dawley rats by a tissue explanting method. Briefly, rats were euthanized *via* intraperitoneal injection of sodium pentobarbital (60 mg/kg) and perfused with PBS for 5 min. Thoracic aorta was aseptically isolated and the surrounding fat and connective tissues were discarded. Vessels were longitudinally cut and the lumen side was scraped with a razor blade to remove the intima. Vessels were incubated with collagenase solution (2 mg/ml) for 30 min and adventitial fibroblast layer was peeled off. The media layer of vessels was fragmented into 3–5 mm lengths and explanted lumen side down on collagen-coated culture dishes. After seven days of explanting, tissue fragments were discarded and sprouted VSMCs were collected (referred as P0). Synthetic type of VSMCs was cultured on gelatin-coated plate at low density (<20%). To induce phenotypic conversion of VSMCs, synthetic type of VSMCs (P0) was cultured on laminin-coated plates at high density (~100%) and passages between P2 and P5 were defined as contractile type of VSMCs.

2.4. Cell proliferation assay and cell cycle analysis

To measure cell proliferation, a total of 2500 VSMCs were seeded on a six-well plate and grown for 5 days in normal culture medium. Cells were fixed with 4% paraformaldehyde, and the nuclei were stained with DAPI. Stained cells were visualized with a fluorescence microscope and random images of four fields were taken at 20× magnification. To analyze cell cycle, a total of 5×10^5 VSMCs were seeded on 100-mm dish and serum-starved for 24 h to synchronize them in the G_1 phase of the cell cycle. Synchronous populations of cells were subsequently treated in the absence or presence of 10% FBS for 18 h. Flow cytometry analysis was performed using FACSCalibur (Becton Dickinson, San Jose, CA). Data were acquired and analyzed with CellQuest software. The percentage of cells in each phase of the cell cycle was calculated as the ratio of events in each phase to the total number of events.

2.5. Collagen gel contraction assay

VSMCs were isolated by trypsin digestion and resuspended in serum-free DMEM (1×10^6 cells/ml). The cell suspension was mixed on ice with collagen gel solution (8 mg/ml of collagen type I in 2× PBS, pH 8.0) to give 5×10^5 cells/ml and 4 mg/ml of collagen gel solution. One hundred microliters of VSMC-collagen gel mixture was added to 12-well plates. The plate was incubated at 37 °C to allow for polymerization. After 30 min, the gels were floated in serum-free DMEM; after 5 h, AngII was added to initiate contraction while images were captured using a digital charge-coupled device camera. Collagen gel contraction was measured as a decrease in gel area using Scion Image software (compliments of Scion Corporation, Frederick, MD;

http://www.scioncorp.com). Relative gel area was obtained by dividing the area at each time point by the initial area of the gel.

2.6. Short hairpin RNA and constructs

To silence Akt1, Akt2, and ILK, oligonucleotides tagged with a 5′-end *AgeI* site and a 3′-end *EcoRI* site were designed for sh-Akt1 (5′-CCG GTA ACT TCT CAG TGG CAC AAT GCC TCG AGG CAT TGT GCC ACT GAG AAG TTT TTT TG-3′), sh-Akt2 (5′-CCG GTG GTC ATC ATT GCA AAG GAT GAC TCG AGT CAT CCT TTG CAA TGA TGA CCT TTT TG-3′), and sh-ILK (5′-CCG GTG ACG CTC AGC AGA CAT GTG GAC TCG AGT CCA CAT GTC TGC TGA GCG TCT TTT TG-3′) and both sense and anti-sense oligonucleotides were synthesized (XENOTECH, Daejeon, Korea). Both complementary oligonucleotides were mixed and heated at 98 °C for 5 min and cooled to room temperature. Annealed nucleotides were subcloned into the *AgeI*/*EcoRI* site of a pLKO.1 lentiviral vector.

2.7. Lentiviral knockdown

For gene silencing, HEK293-FT packaging cells (Invitrogen) were grown to ~70% confluence in 6-well plates. Cells were triple transfected with 6 μg of pLKO.1 lentiviral construct, 1 μg of Δ8.9, and 1 μg of pVSV-G using a calcium phosphate method. Medium was replaced with fresh medium 8 h post-transfection. Lentiviral supernatants were harvested 24 h post-transfection and passed through 0.45-μm filters. Cell-free viral culture supernatants were used to infect synthetic VSMC in the presence of 8 μg/ml of polybrene (Sigma). An additional round of infection was done at 48 h and 72 h post-transfection. Infected cells were isolated by selection with 10 μg/ml puromycin for 2 days.

2.8. Plasmids and promoter assay

To clone myocardin promoter, rat genome was PCR-amplified using forward primer, 5′-CGCTCGAGCAGCTCAGAGGTAGATGGATA-3′; and reverse primer, 5′-CGAAGCTTAGGAGTGTCATGGTGAGTCTC-3′. The XhoI and HindIII restriction enzyme sites are underlined. The promoter fragment was finally cloned into the vector pGL3 basic (Promega). For the measurement of promoter activity, the dual-luciferase reporter assay system was employed. VSMCs were plated in 12-well plates. Cells were co-transfected with the luciferase reporter constructs and the renilla luciferase plasmids using Lipofectamine 2000 (Invitrogen). Each well contained 0.88 μg of luciferase reporter plasmids, 0.8 μg of expression vectors, and 80 ng of renilla luciferase plasmids. Medium was replaced with fresh medium 7 h post-transfection. The cells were lysed and assayed for luciferase activity 24 h after post-transfection. Twenty microliters of protein extracts were analyzed in a GloMax™ 20/20 luminometer (Promega, WI, USA).

2.9. Western blotting and immunocytochemistry

For western blotting, cell lysates were subjected to SDS–polyacrylamide gel electrophoresis on 10% polyacrylamide gel under reducing conditions. Proteins were transferred to nitrocellulose membranes, which were subjected to immunoblotting using the indicated primary antibodies and IRDye-conjugated secondary antibodies (Li-COR biosciences). Western blots were developed using Odyssey (Li-COR biosciences). For immunocytochemistry, cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde for 10 min. Cells were permeabilized with 0.2% Triton X-100, and incubated with indicated primary antibody for 1 h followed by Cy3 or Alexa Fluor 488-conjugated secondary antibody for 30 min. Samples were mounted with anti-fading reagent (2% n-propyl gallate in 80% glycerol/PBS solution) and images were obtained with confocal microscope (FV1000-ZDC, Olympus, Japan).

2.10. Carotid artery ligation, induction of atherosclerosis, and immunohistochemistry

To induce neointima, the left common carotid artery of mouse was ligated proximal to the bifurcation. After 4 weeks, both left and right common carotid arteries were isolated. Atherosclerosis was induced by feeding the mice a high-fat western-type diet containing 1.25% cholesterol (Research Diets, D12108) for 15 weeks. Isolated carotid arteries or aortas were fixed in 4% paraformaldehyde at 4 °C overnight and embedded in paraffin for immunohistochemistry. Five-millimeter sections of each block were stained with hematoxylin & eosin (H&E), or the indicated primary antibodies, and visualized with MIRAX MIDI Versatile Digital Slide Scanner (Carl Zeiss, Jena, Germany) or confocal microscopy (FV1000-ZDC, Olympus, Japan).

2.11. Statistical analysis

Results are expressed as the mean ± SD of three independent experiments ($n = 3$ for each experiment). When comparing two groups, an unpaired Student's *t*-test was used to assess differences. *P*-values less than 0.05 were considered significant.

3. Results

3.1. Phenotypic conversion of VSMCs by laminin

VSMCs exist as two different phenotypes: synthetic phenotype that rapidly proliferates and the contractile phenotype that contracts in response to extracellular stimuli. VSMCs isolated by explanting aortic tissue fragments on collagen-coated plates (P0) showed low expression levels of marker proteins for contractile VSMCs such as MHC, MLCK, SMA, calponin, and SM22α while plating P0 cells on laminin-coated plates initiated the expression of marker proteins for contractile VSMCs (Fig. 1A and B). Phenotypic status was further confirmed by AngII-dependent contraction. As shown in Fig. 1C, AngII rapidly stimulated contraction of P3-stage VSMCs, whereas P0-stage VSMCs did not respond to AngII stimulation.

3.2. Interaction with ECMs is necessary for phenotypic change

Since plating P0 cells on laminin-coated dish induced the expression of smooth muscle marker proteins, we further examined various extracellular matrices (ECMs) on the phenotypic change of VSMCs. As shown in Fig. 2A, plating the synthetic type of VSMCs (60% density) on laminin-coated dish significantly enhanced the expression of SMA and calponin whereas plating the VSMCs on gelatin- or collagen-coated dish did not induce the expression of SMA and calponin. Plating VSMCs on laminin-coated dish (60% density) initiated the expression of SMA and calponin within 24 h (Fig. 2B). In addition, expression of SMA and calponin was increased in a density-dependent manner. Plating the VSMCs on laminin-coated dish at low density (10%) could not induce the expression of SMA and calponin within 24 h. As shown in Fig. 2C, plating synthetic VSMCs on laminin-coated plates rapidly activated both Akt and ERK, whereas gelatin could not induce the activation of Akt and ERK1/2, indicating that laminin-dependent signaling is important for phenotypic change of VSMCs.

3.3. ILK plays an essential role in phenotypic conversion of VSMCs

ILK plays an important role in ECM-mediated signaling pathways. Since the phenotypic conversion of VSMCs requires the interaction with laminin, we examined the role of ILK in the phenotypic conversion of VSMCs. As shown in Fig. 3A, silencing ILK completely abolished basal level of SMA and calponin as well as laminin-dependent expression of SMA and calponin. In addition, plating the VSMC silencing ILK did not show AngII-induced contraction of VSMCs, when compared with vector

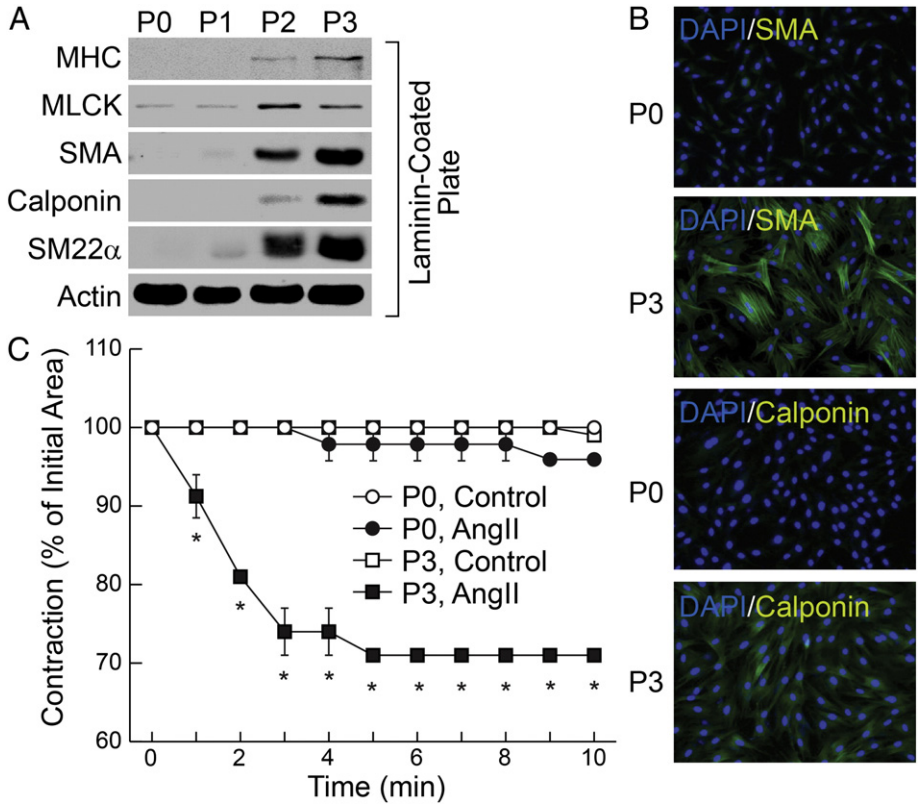

Fig. 1. Phenotypic conversion of synthetic VSMCs into contractile type. Rat aortic tissue fragments were explanted onto collagen-coated plates. After 7 days of culture, sprouting cells were designated as P0 and plated on laminin-coated plates. (A) Expression of marker proteins was verified by western blot analysis; (B) immunocytochemistry at the indicated times. Magnification, 40×; (C) Each stage of cells was embedded in collagen gel bead and stimulated with AngII. Time-lapse image was recorded digitally and contraction was expressed as % of area out of initial area. Data are means ± S.D. Asterisks indicate statistical significance ($P < 0.05$).

transfected control (Fig. 3B). Since attachment of cells with laminin strongly stimulated activation of Akt and ERK, we next examined the effect of ILK on the activation of Akt and ERK. As shown in Fig. 3C–E, silencing ILK significantly attenuated laminin-induced activation of Akt whereas activation of ERK was barely affected.

3.4. Akt1 isoform is required for laminin-mediated phenotypic conversion of VSMCs

Since ILK plays a crucial role in laminin-dependent activation of Akt but not ERK, we examined the effect of Akt and ERK on the phenotypic conversion of VSMCs. As shown in Fig. 4A, pharmacological inhibition of PI3K by LY204002 (50 μM) significantly blocked the expression of SMA and calponin; however, inhibition of ERK signaling pathways by PD98059 (1 μM) enhanced the expression of SMA and calponin. To assess the effect of each Akt isoform on phenotypic conversion of VSMCs, we verified the expression of each isoform and specifically silenced each by lentiviral shRNA expression. As shown in Fig. 4B, both Akt1 and Akt2 isoforms were expressed in VSMCs. Akt1 shRNA effectively silenced Akt1 expression, without significantly affecting Akt2 expression, and *vice versa* for Akt2 shRNA. As shown in Fig. 4C, silencing Akt1 completely blocked the phenotypic conversion of VSMCs whereas silencing Akt2 was not effective. Furthermore, silencing the Akt1 isoform significantly reduced AngII-dependent contraction of VSMCs whereas silencing Akt2 did not affect AngII-dependent contraction (Fig. 4D). Since Akt1 regulates laminin-dependent phenotypic conversion of synthetic VSMCs, we next examined the effect of Akt1 silencing in contractile phenotype of VSMCs on the proliferation. As shown in Fig. 4E, silencing Akt1 significantly induced proliferation of VSMCs whereas silencing of Akt2 did not affect. In addition, silencing Akt1 induced S phase population of the VSMCs (Fig. 4F).

3.5. Akt1 regulates SM22α promoter activity via myocardin expression

Since our results showed that Akt1 was essential for laminin-stimulated conversion of VSMC phenotype, we explored the effect of Akt1 on the promoter activity of myocardin, which is a critical transcriptional factor for the contractile phenotype of VSMCs. As shown in Fig. 5A, promoter activity of myocardin was significantly elevated in the contractile phenotype of VSMCs. In addition, the promoter activity of SM22α, which is a marker protein for the contractile phenotype, was significantly elevated in contractile VSMCs (Fig. 5B). Expression of myocardin in synthetic VSMCs strongly enhanced the promoter activity of SM22α (Fig. 5C). Promoter activity of myocardin and SM22α was significantly abrogated in VSMCs with silenced Akt1 but not Akt2 (Fig. 5D and E). As shown in Fig. 5F, defective promoter activity of SM22α in cells with silenced Akt1 was completely restored by ectopic expression of myocardin. Likewise, the promoter activity of SMA was also regulated by myocardin and Akt (Supplemental Fig. S1).

3.6. Neointima formation is accelerated in mice lacking Akt1

To assess the role of Akt1 in the regulation of VSMC phenotype *in vivo*, the left common carotid artery (LCCA) was ligated distal to the aortic arch near the bifurcation for 4 weeks. As shown in Fig. 6A, neointima formation was significantly augmented in *Akt1*$^{-/-}$ mice compared with *Akt1*$^{+/+}$ mice. Quantitative analysis showed that neointima area was elevated about 2-fold in mice lacking Akt1. Expression of SMA and SM22α was significantly reduced in neointimal lesion compared

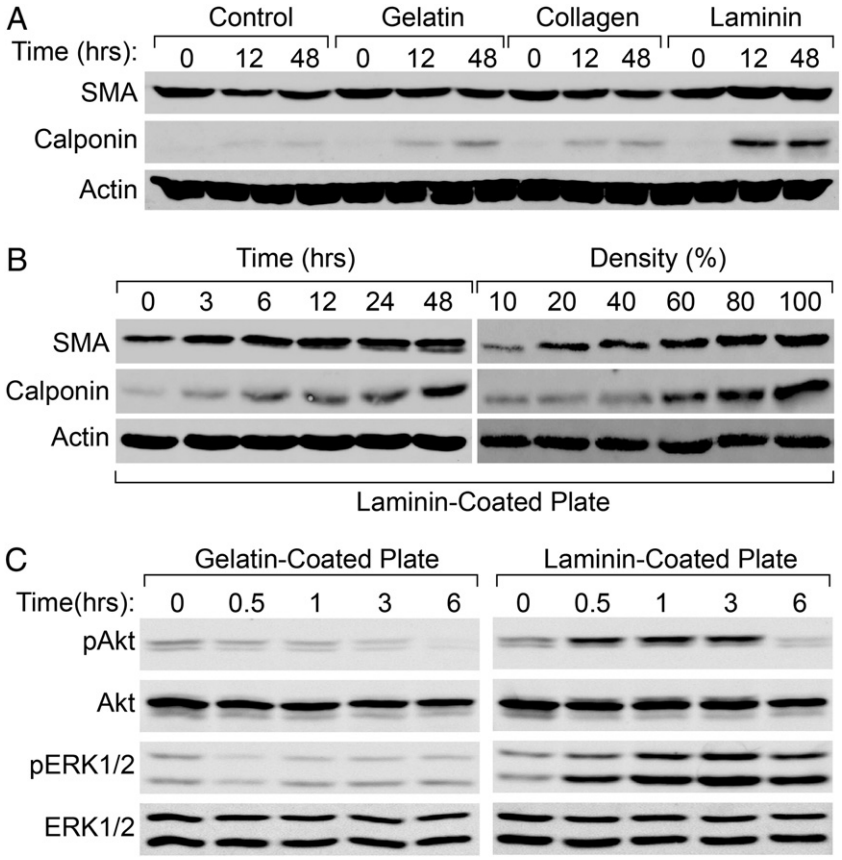

Fig. 2. Laminin-dependent changes of VSMC phenotype. (A) P0 stage of VSMCs was plated on petri-dishes coated with vehicle, gelatin, collagen, or laminin for indicated times. Cells were harvested and expression of SMA and calponin was verified by western blot analysis. (B) P0 stage of VSMCs was plated on laminin-coated dishes and time- and dose-dependent expression of SMA and calponin were examined by western blot analysis.

with medial layer. Since ligation-induced neointima formation is an artificial model, we tried to evaluate the role of Akt1 in a pathological condition such as atherosclerosis. As shown in Fig. 6B, feeding $ApoE^{-/-}Akt1^{-/-}$ mice a high-fat diet (15 weeks) significantly increased number of cells and showed necrotic core in neointima lesion compared with $ApoE^{-/-}Akt1^{+/+}$ mice. In addition, neointima lesion formation in

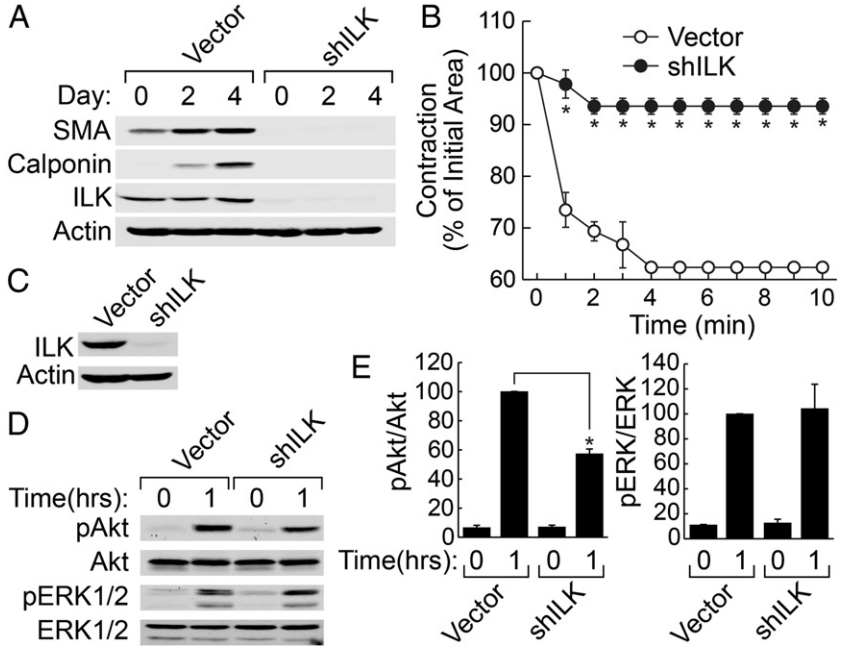

Fig. 3. ILK is necessary for laminin-dependent phenotypic conversion of VSMCs and activation of Akt. ILK was silenced in P0-stage VSMCs. Cells were plated on laminin-coated dishes and further cultured for indicated times. (A) Expression of SMA, calponin, and ILK was verified by western blot analysis; and (B) AngII-dependent contraction was quantified. Data are means ± S.D. Asterisks indicate statistical significance ($P < 0.05$). (C) ILK was silenced in P0-stage VSMCs and expression of ILK was verified by western blotting; (D) activation of Akt and ERK was verified by western blot analysis; and (E) band intensity was quantified by measuring pixel intensity. Asterisks indicate statistical significance ($P < 0.05$).

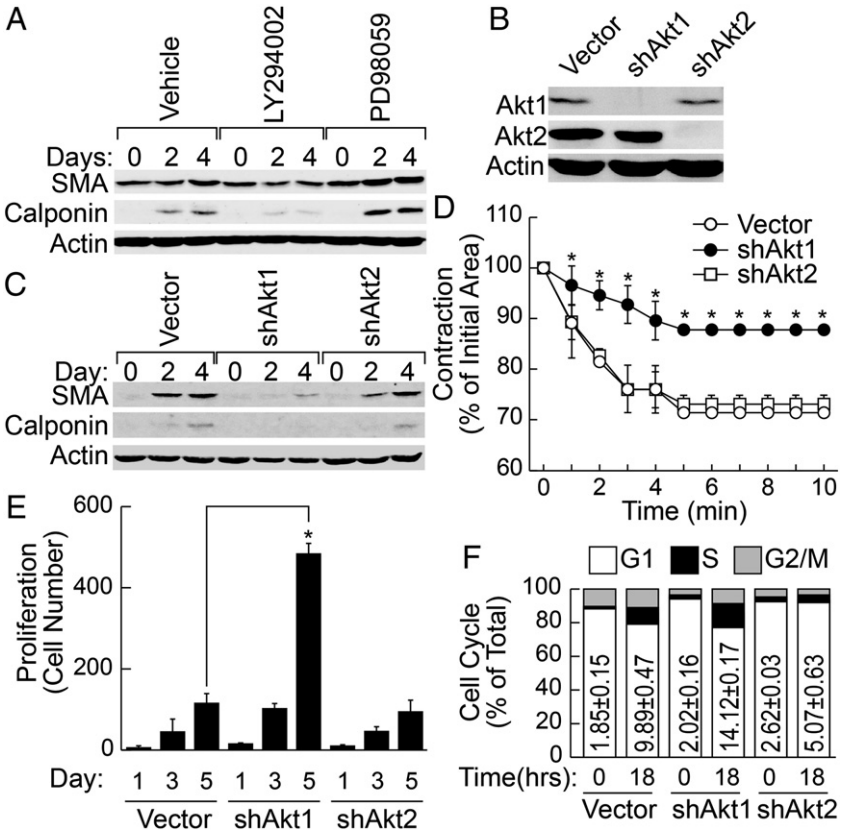

Fig. 4. PI3K/Akt1 is required for laminin-induced phenotypic conversion of VSMCs. (A) P0-stage VSMCs were plated on laminin-coated dishes in the presence or absence of inhibitors. Cells were harvested at the indicated times and expression of calponin and SMA was verified by western blot analysis. (B) Each isoform of Akt was silenced in P0-stage VSMCs and expression of Akt1 and Akt2 was verified by western blot analysis; (C) cells were plated on laminin-coated dishes for the indicated times and expression of SMA and calponin was measured by western blot analysis; and (D) AngII-dependent contraction was quantified. Data are means ± S.D. Asterisks indicate statistical significance ($P < 0.05$). (E) Each isoform of Akt was silenced in P3-stage VSMCs and number of cells was counted at the indicated times. Data are means ± S.D. Asterisks indicate statistical significance ($P < 0.05$); and (F) flow cytometry analysis was performed. Numbers in column represent S phase population.

aortic sinus was significantly enhanced in *ApoE*$^{-/-}$*Akt1*$^{-/-}$ mice (Fig. 6C).

4. Discussion

In the present study, we demonstrated that Akt1 regulates the phenotypic change of VSMCs through laminin–ILK–Akt1 signaling axis-dependent expression of myocardin and smooth muscle marker proteins. In addition, we showed that loss of Akt1 contributed to the induction of intimal thickening *in vivo*. Consequently, we propose that matrix-dependent activation of Akt1 isoform is a potential target for inhibition of intimal thickening.

To a great extent, conversion of synthetic VSMCs into the contractile phenotype resembles the differentiation of proliferating myoblasts into myotubes during the formation of skeletal muscles. However, unlike skeletal muscles, contractile VSMCs are able to switch their phenotype into proliferating VSMCs [19]. Phenotypic switching takes place in atherogenesis, vascular injury, and when vessel fragments are established in culture [7,20]. Indeed, explanting normal vessel fragments onto collagen-coated plate derives descent amount of sprouting cells, which show a proliferative phenotype showing low expression level of marker proteins for contractile phenotype such as MHC, MLCK, SM22α, SMA, and calponin (Fig. 1). This result also suggests that rapidly proliferating VSMCs originates from existing VSMCs in the medial layer of vessels since adventitial layer was discarded.

Many environmental cues have been reported to play a key role in the phenotypic change of VSMCs, including insulin-like growth factor-1 (IGF-1), tumor growth factorβ (TGFβ), mechanical forces, cell-to-cell interaction and ECM proteins [21]. Among ECM proteins, laminin is a major component of the basement membrane that normally surrounds contractile vascular smooth muscle cells. The role of laminin in the phenotypic modulation of VSMCs has also been implicated. For example, laminin was the most effective ECM for maintaining the contractile phenotype of VSMCs [6]. Likewise, our results showed that laminin strongly promoted conversion of the synthetic phenotype of VSMCs into the contractile phenotype (Figs. 1 and 2). In addition, laminin was expressed in medial layer whereas expression of laminin was significantly reduced in neointimal layer indicating that laminin could be an environmental cue for phenotypic change of VSMCs (Supplemental Fig. S2). It is also noteworthy that expression of collagen, which has no effect on VSMCs differentiation, was strongly enhanced in neointimal lesion in comparison with medial layer. Although laminin plays crucial role in phenotypic conversion, it seems not to be the sole factor for the phenotypic conversion. For instance, laminin-induced phenotypic conversion was not observed in the absence of cell-to-cell interaction (Fig. 2). Therefore, the multiple interactions with environmental cues seem to be an important determinant for VSMC phenotype, and the interaction with laminin might be one of the significant inducers for shifting the synthetic phenotype to the contractile phenotype.

Laminins are a family of large heterotrimeric (αβγ) proteins and major isoform of laminin used in this study was laminin-511 (α5β1γ1) [22]. It also has been reported that laminin-511 exists in tunica media [23]. Hence, laminin-511 seems to be important for phenotypic conversion of VSMCs. The binding partner of laminin-511 was not clearly demonstrated in this study, however, it has been reported that laminin-511 preferentially interacts with integrin α3β1 in VSMCs [24,25]. Therefore, it is likely that laminin-dependent activation of β1-integrin signaling pathways is important for modulating phenotypic

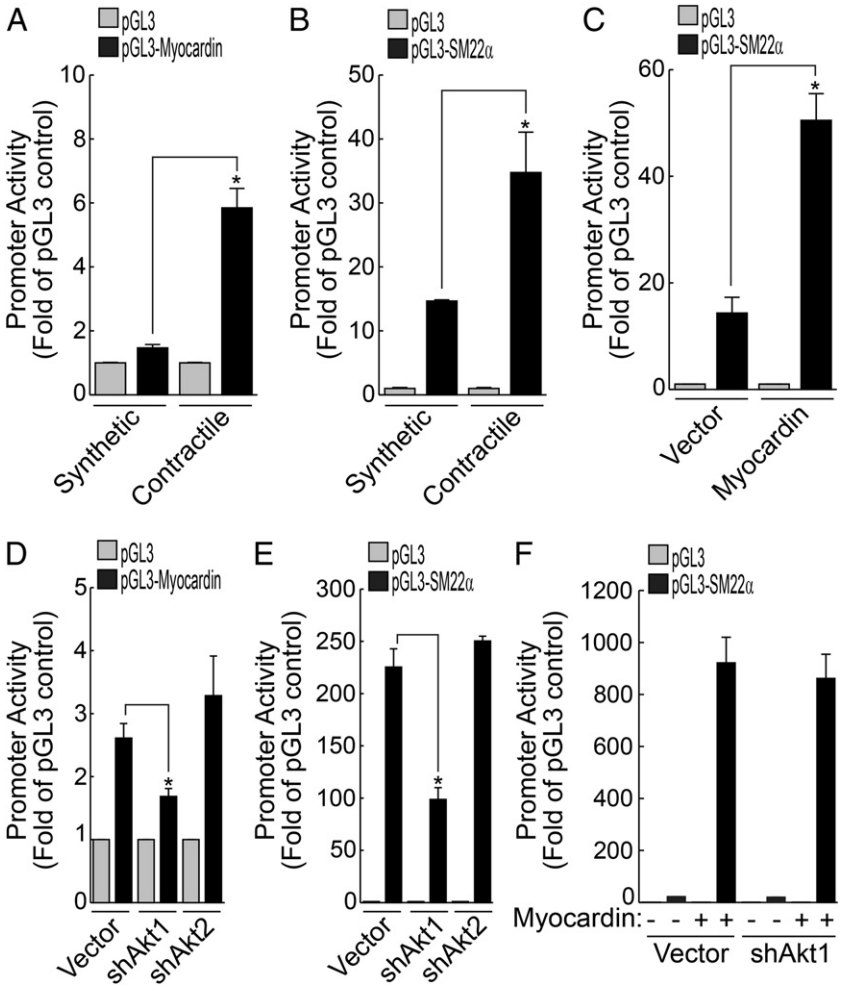

Fig. 5. Akt1 isoform is required for the expression of myocardin and subsequent expression of SM22α. (A) Luciferase-tagged promoter was transfected into P0 (synthetic)- or P3 (contractile)-stage VSMCs, and luciferase activity was measured for promoter activity of myocardin; and (B) promoter activity of SM22α. (C) Promoter activity of SM22α was measured in P0 (synthetic)-stage VSMCs in the presence or absence of myocardin expression. (D) Either Akt1 or Akt2 was silenced in P3 (contractile)-stage VSMCs and luciferase activity was measured for promoter activity of myocardin; and (E) promoter activity of SM22α. (F) Akt1 was silenced in P3 (contractile)-stage VSMCs and promoter activity of SM22α was measured in the presence or absence of myocardin expression. Data are means ± S.D. of three independent experiments ($n = 3$ for each experiment). Asterisks indicate statistical significance ($P < 0.05$).

conversion of VSMCs. ILK was originally identified as β1-integrin binding protein in yeast two-hybrid screen [12]. Indeed, ILK seems to be a key regulatory molecule for laminin-induced phenotypic conversion of VSMCs (Fig. 3). Several reports support the pivotal role of ILK in VSMCs. For example, ILK plays a crucial role in the regulation of VSMC physiologies such as adhesion, migration, and proliferation [26]. In addition, conditional deletion of ILK in smooth muscle resulted in alteration of smooth muscle marker gene expression [27], indicating that ILK signaling pathway is linked to the expression of contractile smooth muscle marker genes. In line with this, laminin-induced expression of marker proteins for contractile phenotype of VSMCs such as SMA and SM22α as well as AngII-dependent contraction was completely abolished in cells with silenced ILK (Fig. 3). The laminin–integrin–ILK signaling axis therefore seems to be important for converting VSMCs from the synthetic phenotype to the contractile phenotype as well as for maintaining contractile phenotype of VSMCs.

One of the downstream targets of ILK is Akt serine/threonine kinase, which is involved in a variety of cellular physiologies. ILK activates Akt by phosphorylation at Ser473 [13]. Likewise, our results showed that attachment of VSMCs on laminin-coated plates, but not collagen-coated plates, rapidly induced the phosphorylation of Akt (Fig. 2), and silencing of ILK resulted in the attenuation of Akt phosphorylation (Fig. 3). It is also noteworthy that activation of ERK was not affected by silencing of ILK, indicating that the activation of ERK is independent of ILK activation (Fig. 3D and E). Pharmacological inhibition of PI3K significantly abrogated phenotypic change of VSMCs. In contrast, inhibition of the ERK stimulated phenotypic change of VSMCs (Fig. 3A). It is still unclear how inhibition of ERK signaling pathway accelerates laminin-induced phenotypic change of VSMCs, however, it has been reported that the balance between PI3K/Akt and ERK signaling pathways is important for maintaining the contractile phenotype of VSMCs. For instance, strong activation of the ERK signaling pathway by platelet-derived growth factor (PDGF) rapidly promotes the phenotypic change of VSMCs from contractile to synthetic [28]. Therefore, it is reasonable that inhibition of the ERK signaling pathway would accelerate the phenotypic change from synthetic to contractile.

In the present study, we emphasized the isoform-specific regulation of the VSMC phenotype. Both Akt1 and Akt2 isoforms existed in VSMCs (Fig. 4B). However, the laminin-induced phenotypic change was only affected by silencing Akt1 but not Akt2 (Fig. 3C and D). In correlation with phenotypic changes, promoter activity of myocardin and SM22α was selectively attenuated by silencing Akt1 but not Akt2 (Fig. 5D and E). In addition, proliferation of contractile phenotype of VSMCs was selectively facilitated by silencing Akt1 but not Akt2 (Fig. 4E and F). On the other hand, it has been reported that glucose transporter 4 (GLUT4) translocation and glucose uptake are mediated in an Akt2-specific manner [29]. Taken together, it is likely that Akt1 isoform has distinct functions in the regulation of phenotypic changes of VSMCs. However, it is

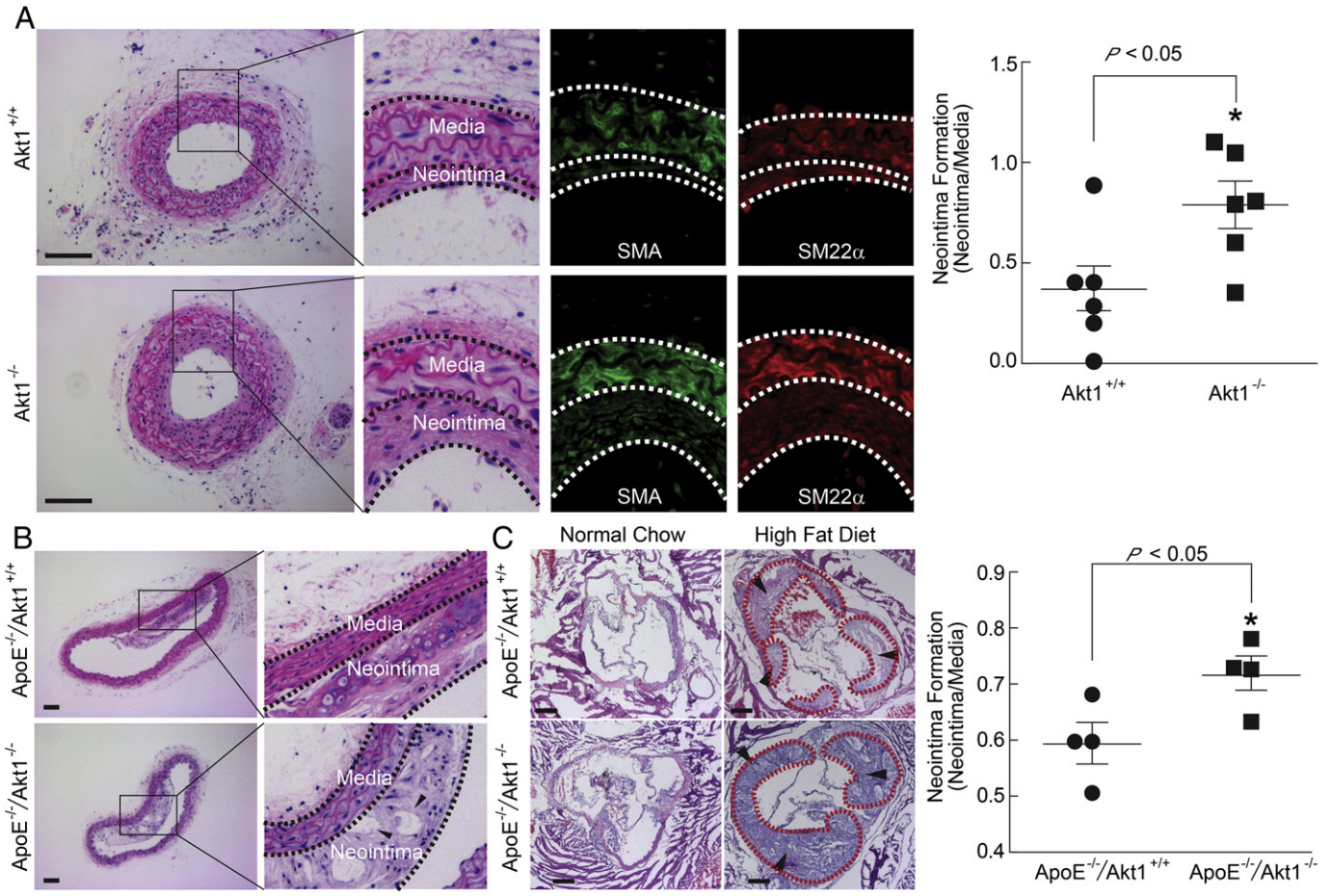

Fig. 6. Loss of Akt1 enhances intimal hyperplasia. (A) Carotid arteries of mice were ligated for 4 weeks. Left: representative photomicrographs of cross-sections from the carotid arteries stained with hematoxylin and eosin, SMA, and SM22α. Dashed line defines media and neointima layer. Bar, 50 μm; right: quantitative analysis of neointima area. Results are presented as the ration of neointimal area to media area. n = 6 for each group. (B) Mice were fed a high-fat diet for 15 weeks. Representative photomicrographs of cross-sections from the aortic arch stained with hematoxylin and eosin. Dashed line defines media and neointima layer. Arrows indicate necrotic core. Bar, 50 μm; (C) Left: representative photomicrographs of cross-sections from the aortic sinus stained with hematoxylin and eosin. Arrows indicate neointima. Bar, 100 μm; right: quantitative analysis of neointima area. Results are presented as the ration of neointimal area to media area. n = 4 for each group.

still unclear how Akt1 isoform regulates phenotypic conversion of VSMCs. One possible mechanism could be related with the regulation of myocardin expression or its transcriptional activity (Fig. 5). In line with this, it has been reported that Akt modulates VSMC phenotype through regulation of FoxO4 with myocardin [30]. However, isoform specific function of Akt in this regulation remains to be elucidated.

The involvement of Akt1 in the process of vascular remodeling has been implicated *in vivo*. It has been reported that loss of Akt1 increases vessel wall inflammation to enhance atherosclerosis [31,32]. In addition to this, we showed that the major population of cells in the neointima area was not detected by antibodies against SMA and SM22α, indicating that most of those cells were synthetic VSMCs (Fig. 6A). We also showed that infiltration of cells into the fibroblast cap lesion significantly enhanced in mice lacking Akt1 (Fig. 6B). Therefore, it is likely that Akt1 might regulate phenotypic conversion during the neointima formation *in vivo*.

In terms of ILK and Akt function, discrepancies remain between proliferation and neointima formation. It has been reported that suppression of ILK resulted in the inhibition of neointima formation as well as in the activation of Akt [33]. In line with this, our results showed that silencing ILK severely blocked proliferation of VSMCs (data not shown). It is also notable that silencing ILK resulted in complete loss of marker protein for contractile phenotype of VSMCs at the basal level while the activation of Akt was moderately affected (Fig. 3). A simple hypothesis would be the suppression of neointima formation by ablation of ILK since ILK is important for cell proliferation and migration. However, loss of Akt1 in contractile phenotype of VSMCs clearly enhanced proliferation (Fig. 4E and F). In addition, loss of Akt1 facilitated neointima formation [31]. In line with this, our result showed intimal hyperplasia in mice lacking Akt1, and these cells were not characterized as contractile VSMCs (Fig. 6). The plausible interpretation for these discrepancies could be deduced from cellular context. In synthetic phenotype, loss of Akt1 fails to acquire contractile phenotype and remains synthetic phenotype which shows faster proliferation rate than contractile phenotype. In contractile phenotype, loss of Akt1 makes VSMCs susceptible to phenotypic conversion into synthetic phenotype and acquires intimal hyperplasia. It is possible that another isoform of Akt replaces the function of Akt1 in the regulation of VSMC proliferation in both cases. In this regard, ILK seems to be required for the proliferation of VSMCs unlike Akt1.

In conclusion, laminin-induced activation of Akt1 isoform plays a pivotal role in the expression of contractile marker proteins and the maintenance of the contractile phenotype of VSMCs. Our findings support the notion that specific signaling pathway of Akt1 functions as a regulator of neointima formation. Therefore, Akt1 may be an important therapeutic target for occlusive arterial diseases such as restenosis and atherosclerosis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.08.014.

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